

EXPRESS MAIL LABEL NO. EV077493871US

JC07 Rec'd PCT/PTO 18 MAR 2002

Form PTO-1390 (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 410718.90395
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			US. APPLICATION NO. (If known, see 37 CFR 1.51) 10/088405
INTERNATIONAL APPLICATION NO. PCT/CA00/01132	INTERNATIONAL FILING DATE 21 Sept 2000 (21.09.00)	PRIORITY DATE CLAIMED 21 Sept 1999 (21.09.99)	
TITLE OF INVENTION LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY			
APPLICANT(S) FOR DO/EO/US CHANDRASEKAR, Baskaran; TANGUAY, Jean-Francois			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 			
Items 11. to 16. below concern document(s) or information included:			
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98 and Form 1449.			
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.			
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.			
14. <input type="checkbox"/> A substitute specification.			
15. <input type="checkbox"/> A change of power of attorney and/or address letter.			
16. <input checked="" type="checkbox"/> Other items or information: Copy of Form PCT/IB/308 dated 29 March 2001 Confirming Transmittal of the International Application to the US as Designated Office; Postcard			

1C10 Rec'd PCT/PTO 1 8 MAR 2002

U.S. APPLICATION NO. (If known, see 37 CFR 1.51)		INTERNATIONAL APPLICATION NO. PCT/CA00/01132		ATTORNEY'S DOCKET NUMBER 410718.90395	
10/088405					
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00				CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	8 -20 =	0	X \$18.00	\$	
Independent claims	1 -3 =	0	X \$78.00	\$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$	
TOTAL OF ABOVE CALCULATIONS =				\$890.00	
<input checked="" type="checkbox"/> Applicant hereby claims small entity status. Reduction by 1/2 for filing by small entity.				\$445.00	
SUBTOTAL =				\$445.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.429(f)).				+ \$	
TOTAL NATIONAL FEE =				\$445.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				+ \$	
TOTAL FEES ENCLOSED =				\$445.00	
				Amount to be refunded	\$
				Charged	\$
a. <input type="checkbox"/> A check in the amount of \$ <u> .00 </u> to cover the above fees is enclosed.					
b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>17-0055</u> in the amount of \$445.00 to cover the above fees. A duplicate copy of this sheet is enclosed.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>17-0055</u> . A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: Quarles & Brady LLP 411 East Wisconsin Ave. Milwaukee, WI 53202-4497			<div style="font-size: 24pt; font-family: cursive;">Jean C. Baker</div> _____ SIGNATURE Jean C. Baker _____ NAME <u>35,433</u> REGISTRATION NUMBER		

2. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 $\mu\text{p/Kg}$ of patient's body weight.
3. The use, as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 50 $\mu\text{p/Kg}$ of patient's body weight.
4. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 30 $\mu\text{p/Kg}$ of patient's body weight.
5. The use as defined in claim 1, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).
6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.
7. The use as defined in claim 4, wherein 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.
8. The use as defined in claim 1, which is for a single administration.

Remarks

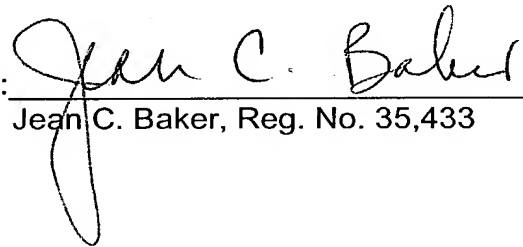
The above amendments are being made to eliminate multiple dependencies in the claims of this application.

No fee is believed necessary to enter this amendment. However if a fee is necessary, please charge Deposit Account 17-0055.

Applicant respectfully requests that the preliminary amendment described herein be entered into the record prior to examination and consideration of the above-identified application.

QUARLES & BRADY LLP

BY:



Jean C. Baker, Reg. No. 35,433

Date: March 18, 2002

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6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.

7. The use as defined in claim 4, wherein 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.

8. The use as defined in [any one of claims 1 to 7] claim 1, which is for a single administration.

TITLE OF THE INVENTION

Local Delivery of 17-beta Estradiol for Preventing vascular
intimal hyperplasia and for improving vascular endothelium function after
5 vascular injury

FIELD OF THE INVENTION

The present invention relates to the local use of estradiol or
10 a derivative thereof to improve the outcome of a coronary angioplasty. More
specifically, the present invention is concerned with the local use of estradiol
or a derivative thereof for decreasing neointima hyperplasia that occurs
during restenosis, and for improving the endothelium function after vascular
injury, both events contributing to the ultimate success of an angioplasty.

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BACKGROUND OF THE INVENTION

Restenosis is currently the major limitation of percutaneous
transluminal coronary angioplasty (PTCA), and is seen in up to 30-40 % of
20 patients.¹ The most important mechanisms contributing to restenosis are
neointima proliferation, vascular remodelling, and elastic recoil.² Elastic recoil
and vascular remodelling can be reduced to a large extent by stenting.³
Although radiation therapy has been reported to show beneficial effects,^{4,5}
no effective therapy exists yet for neointima proliferation. Vascular smooth
25 muscle cell (SMC) migration and proliferation have been documented to
occur as early as 36 hours following arterial injury.⁶ In cell culture assays, 17-
beta estradiol inhibited migration and proliferation of rat vascular SMC.^{7,8}
Similar effects have also been shown with human vascular SMC from
saphenous vein.⁹ Prolonged systemic administration of estrogen has been
30 shown to inhibit intima hyperplasia in animal studies.^{10,11} Instead of

Other objects, advantages and features of the present invention will become more apparent upon reading of the following nonrestrictive description of preferred embodiments thereof, given by way of examples only, with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Representative light micrographs (x 40 magnification) of arterial segments from the same animal, stained with Verhoeff's stain. 17-beta estradiol (a) treated segment shows markedly less neointima hyperplasia compared to PTCA only (b), or vehicle alone (c) groups. The extent of injury is similar in all 3 segments.

Figure 2 Comparison of (A) neointima area, (B) neointima/media area, (C) restenotic index, and (D) % stenosis between PTCA alone vs vehicle only, and PTCA only vs 17-beta estradiol groups; * $p < 0.05$, ** $p < 0.01$ *** $p < 0.002$. Values are expressed as mean \pm SEM.

Figure 3 Representative coronary angiograms demonstrating the vasoconstrictive response to intracoronary infusion of acetylcholine (Ach) 10^{-4} M obtained from the same animal at 4 weeks following percutaneous transluminal coronary angioplasty (PTCA). Column A = basal, column B = after Ach, column C = following intracoronary nitroglycerin. Top panel = treatment with vehicle, mid panel = PTCA only, lower panel 17-beta estradiol treatment groups respectively.

Figure 4 Representative light micrographs (x 1000) of cross sections of vessels obtained from the same animal for immunohistochemical staining with the lectin *Dolichos biflorus* agglutinin (evident as dark brown staining of luminal surface). Vessels treated with 17-beta estradiol (A) demonstrate reendothelialization to a greater degree as compared to PTCA only (B) and vehicle (C) groups.

Figure 5 Representative light micrographs (x 1000) of cross sections of vessels obtained from the same animal, for immunohistochemical analysis

of endothelial nitric oxide synthase (eNOS) expression. Vessels treated with 17-beta estradiol (A) show greater expression of eNOS (evident as dark brown staining of luminal surface) as compared to PTCA only (B) and vehicle (C) groups.

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Figure 6 Graph depicting correlation between vasoconstrictive response to Ach 10^{-4} M and (A) reendothelialization ($r = -0.48$, $p < 0.02$), (B) eNOS expression ($r = -0.58$, $p < 0.005$). Note: % vasoconstriction denotes % decrease in diameter following Ach 10^{-4} M as compared to the basal diameter.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

Example 1: The effect of estradiol on neointima hyperplasia

15 **Methods**

Animal preparation

Eighteen juvenile farm pigs (9 female, and 9 castrated male) weighing 20-25 kg were studied. The study was approved by, and conducted in accordance with, the guidelines of the Animal Care and Ethical Research Committee of the Montreal Heart Institute. Before the procedure, animals were given 650 mg of acetylsalicylic acid and 30 mg of nifedipine orally, premedicated with intramuscular injection of 6 mg/kg of a mixture of tiletamine hydrochloride and zolazepam hydrochloride, and given 0.05 mg of atropine. The invasive procedure was performed under general anesthesia with a mixture of isoflurane (1 to 1.5 %) and oxygen enriched air. The right femoral artery was cannulated percutaneously, and an 8 Fr arterial sheath was introduced. After arterial access had been obtained, 100 mg of lidocaine and 250 U/kg of heparin were administered intra-arterially via the sheath. Activated

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coagulation time was maintained at > 300 seconds throughout the procedure.

Preparation of estradiol formulation

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Each dose individually administered to the tested animals is composed of at least 12.5 mg hydroxypropyl-beta-cyclodextrin (HPCD) and 600 µg estradiol in a 5 ml solution volume.

- 10 A smaller or larger dose may be used. Indeed, the tested dose corresponds to the dose of about 675 µg formulated in a sublingual pellet and administered to postmenopausal women.⁴⁵ Such a dose may be unnecessarily high if administered locally. Indeed, doses of 200 and 400 µg have been tried and they were found to be as performing as the dose of
- 15 600 µg. Further, the necessary dose for performing the present invention may be influenced by the hormonal balance of the individual to be treated. Species variance is also a factor affecting the dosage regimen. Also, any derivative of 17-beta estradiol may replace the latter. A derivative is intended to cover a precursor, an active metabolite, an active analog or a
- 20 modulator capable of positively influencing the activity of the receptor(s) to estradiol or of enhancing the binding and/or the activity of estradiol towards its receptor(s). Such derivatives are considered functional equivalents of 17-beta-estradiol, and therefore within the scope of this invention. A unit dose of 1 to 5000 µg/Kg of 17-beta-estradiol or an equivalent derivative dose is
- 25 within the scope of this invention, preferably 10-50 µg/Kg, even more preferably 10-30 µg/Kg.

Angioplasty and Local Delivery

Standard PTCA equipment was used. An 8 Fr right Amplatz guiding catheter and right Judkins guiding catheter were used for cannulation of the left and right coronary arteries, respectively. PTCA was performed with a balloon size chosen to correspond to a balloon/artery ratio of 1.1-1.3. Three 30-second inflations at 10 atm pressure were performed with a 30-second interval between each inflation. Inflations were performed adjacent to major side branches to facilitate identification during harvesting, taking precaution not to include any side branch in the intended PTCA site. The left anterior descending, left circumflex, and right coronary arteries of each animal were subjected to PTCA. After PTCA, each coronary artery of an animal was randomized to receive either 600 µg of 17-beta estradiol locally, or vehicle alone locally, or PTCA only. The chemicals 17-beta estradiol and its vehicle 2-hydroxypropyl-beta-cyclodextrin (HPCD) were purchased from Sigma Chemical Co. The InfusaSleeve catheter (Local Med, Inc.) was used for local delivery.¹² Five ml of the designated substance was delivered at a driving pressure of 10 atm and support balloon pressure of 6 atm.

Of the 18 animals, 2 died a few days after PTCA, and were excluded; thus, 16 animals were analyzed. Twelve animals were euthanized at 28 days, and 4 at 7 days. After premedication and anesthesia, the right internal jugular vein and common carotid artery were cannulated. Following cross-clamping of the descending thoracic aorta exposed via a left lateral thoracotomy, exsanguination was performed, with simultaneous administration of 1 l of 0.9 % NaCl solution. The heart was perfusion-fixed in vivo with 2 l of 10 % buffered formalin at 200 mm Hg pressure, removed from the animal, and placed in 10 % buffered formalin solution. Coronary arteries were then dissected free from surrounding tissues. The site of PTCA was identified in relation to adjacent side branches, which served as landmarks. The injured

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Immunohistochemistry

Following slicing with a microtome and blocking of non-specific antibodies, the sections were treated with mouse anti-proliferating cell nuclear antigen (PCNA) antibodies and diluted biotinylated goat anti-mouse antibodies. They were then incubated with avidin-biotin (Elite ABC Kit, Vector Laboratories), and developed with 3, 3'-diaminobenzidine (Vector Laboratories). They were finally counter-stained with hematoxylin. Porcine liver cells were used as a positive control. For each section, a 6 μ m slice counter-stained with hematoxylin without treatment with the primary antibody (mouse anti- PCNA) served as a negative control.

The proliferative response to injury was studied by immunohistochemical analysis of samples from animals euthanized at 7 days. The % proliferating SMC was obtained by dividing the number of PCNA - positive SMC by the total number of SMC in each field; separate measurements were made for neointima and media layers. The proliferating cells were identified as SMC by positive staining of parallel sections with a smooth muscle actin antibody. To standardize comparison among treatment groups, measurements were obtained at 4 fixed locations separated by 90° sites for each section, and the results averaged. For each segment, two sections demonstrating maximal neointima response were analyzed, and the results averaged.

Statistical Analysis

Values are expressed as mean \pm standard deviation, except as otherwise indicated. Kruskal - Wallis analysis was used for comparison of data among the 3 groups; subsequently, 17-beta estradiol and vehicle alone groups were separately compared with the PTCA only group using the Mann - Whitney rank sum test. Chi - square analysis was used for comparison of proportions.

Of the 12 animals that underwent morphometric analysis at 28 days, arterial segments treated with local delivery of 17-beta estradiol showed significantly less neointima hyperplasia (Figure 1). This beneficial effect was noted in all parameters of neointima response to injury that were analyzed (Table 1). Of note, the extent of morphologic injury was similar among the 3 groups, suggesting that the use of the InfusaSleeve catheter was not associated with an enhanced risk of injury.

It was important to exclude an inhibitory effect on intima proliferation due to the vehicle, and, to confirm that the effect noted was in response to treatment with 17-beta estradiol. Analyses comparing segments treated with vehicle alone and PTCA only showed a similar response in terms of the extent of neointima proliferation. On the other hand, significantly less intima hyperplasia was observed in 17-beta estradiol treated segments as compared to segments treated with PTCA only (Figure 2). Compared to PTCA only, or vehicle alone, 17-beta estradiol decreased neointima formation by 54.6 % and 64.9 % respectively.

To exclude the possibility of influence of sex on response to estrogen, the 7 segments obtained from male pigs treated with 17-beta estradiol, and 5 segments obtained from female pigs treated with 17-beta estradiol were analyzed. No statistically significant differences were evident (Table 2).

Immunohistochemistry

The number of PCNA - positive SMC was low overall; sacrifice at an earlier time might have yielded a higher number. However, a statistically significant decrease in the proliferative response was seen in animals treated with 17-beta estradiol. Among the different groups, the % of PCNA - positive SMC in the neointima were 0.43 ± 0.52 % in 17-beta estradiol, 4.26 ± 2.33 % in PTCA only, and 4.27 ± 2.73 % in vehicle alone groups respectively ($p < 0.05$ for 17-beta estradiol vs other 2 groups). There were no statistically significant differences in % PCNA - positive SMC in the media among the 3 groups: 0.4 ± 0.3 %, 1.38 ± 1.74 %, and 1.24 ± 1.57 % for 17-beta estradiol, PTCA only, and vehicle alone groups respectively ($p = \text{NS}$).

Vascular remodeling

To determine the effect on vascular remodeling of the agents used, the EEL area of the injured segment and of the normal vessel proximal to site of PTCA were obtained, and their ratio calculated.¹³ No significant difference among the groups was noted: 1.01 ± 0.16 , 1.16 ± 0.28 , 1.31 ± 0.37 respectively for 17-beta estradiol, PTCA only, and vehicle alone groups respectively (p = NS).

10 Conclusions

The present study demonstrates, for the first time, that locally delivered 17-beta estradiol decreases neointima proliferation following PTCA in pigs. The study also shows that the InfusaSleeve catheter can be used to deliver effectively 17-beta estradiol intramurally in coronary arteries.

Several previous experiments in animals have demonstrated that estrogen administered subcutaneously for up to 3 weeks inhibited the myointima response to arterial injury.^{10,11} Recently, short-term subcutaneous estrogen therapy (6 to 17 days) was also shown to be effective in reducing the injury response in rat carotid artery.¹⁶ Estrogen administered intra-muscularly for at least 3 weeks has also demonstrated the potential to inhibit vascular smooth muscle cell proliferation and neointima hyperplasia in rabbits.¹⁷ However, the efficacy of local delivery of 17-beta estradiol to inhibit intima hyperplasia has not been previously studied.

The biologic effects of estrogen, like other steroid hormones, involve intracellular receptors. The first estrogen receptor (ER) to be discovered was ER α ,^{18,19} which was thought to mediate the beneficial effects of estrogen following vascular injury. ER α was also present in coronary arteries obtained

from autopsy specimens in both pre and postmenopausal women,²⁰ and in cell cultures of human saphenous vein and internal mammary artery specimens.²¹ Recently, a second estrogen receptor, ER β , has been identified in animals and humans.^{22,23} The role of ER β in response to vascular injury was subsequently demonstrated in experiments with ER α deficient mice.²⁴ Normal and ER α deficient mice treated with estrogen, when subjected to arterial injury, showed the same extent of inhibition of neointima proliferation compared to control mice; thereby demonstrating that inhibition of vascular injury response by estrogen is independent of ER α . Although the present experiment was not designed to study the mechanism of action of 17-beta estradiol, evidence exists for multiple potential mechanisms by which 17-beta estradiol can inhibit the vascular response to injury. Of importance may be the effect of 17-beta estradiol on nitric oxide (NO) synthesis. In cell culture studies with human and bovine endothelial cells, treatment with 17-beta estradiol stimulated NO synthase and increased NO production.^{25,26} Postmenopausal women treated with transdermal 17-beta estradiol showed enhanced in vivo NO synthesis.²⁷ NO has demonstrated inhibitory effects on both migration²⁸ and proliferation²⁹ of vascular SMC, and decreased neointima formation after PTCA.¹³ Preliminary reports have shown that therapy with 17-beta estradiol decreases intercellular and vascular cell adhesion molecule expression by human coronary SMC.³⁰ Cellular adhesion molecules are expressed by SMC following arterial injury³¹ and their suppression with the use of monoclonal antibodies inhibited intima hyperplasia after arterial injury in rats.³² The regulatory effect of 17-beta estradiol on vascular endothelial growth factor expression may also be partly responsible.³³⁻³⁵ Perhaps the most important mechanism may be a direct inhibitory effect of 17-beta estradiol on vascular SMC proliferation.³⁶ The binding of 17-beta estradiol to its intracellular receptor activates DNA containing "estrogen responsive elements", leading to altered gene

expression. 17-beta estradiol also reduces platelet derived growth factor-induced migration and proliferation of vascular SMC.⁹

The beneficial effects of 17-beta estradiol, the predominant circulating estrogen in premenopausal women, on vascular injury response may not be replicated by other kinds of estrogens; for example, conjugated equine estrogen was found to have no effect on neointima proliferation in non-human primate models.³⁷ Simultaneous administration of progesterone may attenuate the vascular injury response to 17-beta estradiol.³⁸ A sexually dimorphic response to estrogen in intact rats has been reported following arterial injury, with male rats deriving no benefit with estrogen therapy.³⁹ This sexually dimorphic effect was, however, not observed in another experiment with gonadectomized rats.¹¹ In the present study, too, no significant difference in neointima proliferative response to 17-beta estradiol was noted between the sexes. Increased expression of ER β mRNA (ER β is directly associated with inhibition of vascular SMC proliferation) following arterial injury has been demonstrated in intact male rats;⁴⁰ of additional interest in the study is that no increase in ER α was seen following arterial injury.

17-beta estradiol is a lipophilic compound with poor solubility in aqueous solutions, thereby needing a vehicle for parenteral administration. HPCD is a starch derivative that has been successfully tested as an effective excipient for protein drugs.⁴¹ The pharmacokinetics of HPCD are similar to that of inulin, and the toxic dose (nephrotoxicity) has been estimated to be 200 mg/kg in rats.⁴² The dose of HPCD used to dissolve 17-beta estradiol in the present study was 0.63 mg/kg, far below the toxic dose. Furthermore, HPCD has been used for administration of ophthalmic preparations and intravenous anaesthetic agents in humans.^{43,44} HPCD complexed to 17-beta estradiol has been used to enhance bioavailability of orally, or, sublingually administered 17-beta estradiol with no untoward effects in humans.⁴⁵

- Retrospective studies in humans have shown no benefit of hormonal replacement therapy on angiographic restenosis following PTCA⁴⁶ although one study did show a beneficial effect after directional atherectomy.⁴⁷
- 5 However, it should be noted that conjugated estrogen (and not 17-beta estradiol) was the predominant form of estrogen used in many of these patients, and, no information about concomitant use of progesterone is available.
- 10 In conclusion, we have shown that, a single dose of 17-beta estradiol delivered locally during PTCA has the potential to inhibit neointima proliferation effectively. The delivery of 17-beta estradiol can be performed easily with the InfusaSleeve catheter, without risk of additional injury. With this approach, it may be possible to avoid potential undesirable effects of
- 15 long term systemic administration of estrogen. ER β has been identified in humans, and inhibition of proliferation of human vascular SMC by 17-beta estradiol has been demonstrated in cell culture assays. The local administration of 17-beta estradiol is therefore a promising new approach, which might be useful in preventing the proliferative response after PTCA in
- 20 humans. Its usefulness in preventing restenosis after PTCA is contemplative in view of the foregoing promising results.

Example 2: The effect of estradiol on vascular endothelial function

Methods

25 Animal preparation

The study protocol was approved by the Animal Care and Ethical Research Committee of the Montreal Heart Institute. Juvenile farm pigs weighing 20-25 kg (1 female, and 8 castrated males) were used. On the day of the

30 experiment, animals received 650 mg of acetylsalicylic acid and 30 mg of

nifedipine orally, were premedicated with 6mg/kg of tiletamine hydrochloride and zolazepam hydrochloride, and were given 0.05 mg of atropine intramuscularly. Under general anesthesia (a mixture of 1-1.5% isoflurane and oxygen enriched air), the right femoral artery was cannulated percutaneously. An 8 Fr arterial sheath was introduced, and 100 mg/kg of lidocaine and 250 U/kg of heparin were administered intra-arterially. Additional heparin was administered during PTCA if needed, to maintain an activated coagulation time of > 300 seconds.

10 Procedure

An 8 Fr right Amplatz guiding catheter and right Judkins guiding catheter were used for cannulation of the left and right coronary arteries, respectively. A standard balloon catheter (corresponding to a balloon/artery ratio of 1.1-1.3: 1) was advanced over a 0.014" floppy guide wire, and 3 successive 30-second inflations at 10 atm pressure were made with a 30 second interval between each inflation. PTCA was performed on all 3 coronary arteries of each animal. For local delivery, the InfusaSleeve catheter (LocalMed Inc.) was used, which permits safe drug delivery with negligible additional injury (7). After balloon dilatation, each coronary artery of an animal was randomized to receive either 600 µg of 17-beta estradiol (in 5 ml), vehicle alone (5 ml), or PTCA only. The vehicle 2-hydroxypropyl-beta-cyclodextrin (HPCD), and 17-beta estradiol were obtained from Sigma Chem. Co. For local delivery with the InfusaSleeve catheter, a proximal driving pressure of 10 atm and support balloon pressure of 6 atm were utilized.

Intracoronary infusion

All 9 animals underwent cardiac catheterization at the end of 4 weeks. After a baseline coronary angiogram, selective cannulation of the proximal portion

of a coronary artery was performed with a single lumen balloon catheter (TotalCross, Schneider) for the administration of vasoactive agents. Acetylcholine (Ach) in increasing concentrations of 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, was successively infused through the lumen port of the catheter.

5 Each dose was administered for a duration of 3 minutes at a constant rate of 1 ml/min using an infusion pump. Coronary angiography was performed at the end of each dose. After infusion of the highest concentration of Ach (10^{-4} M and angiography, 100 μ g of nitroglycerin was administered via the lumen port of the catheter, and a coronary angiogram performed. The same

10 protocol was repeated for the other 2 coronary arteries. Heart rate, blood pressure, and ECG were monitored continuously throughout the experiment.

Quantitative coronary angiography

15 Coronary angiography was performed with a single plane imaging system (Electromed Intl). Images were obtained in predetermined views which best demonstrated the vessel segment of interest and without overlap of branches. Care was taken to maintain the same angulation during angiography of a segment throughout the procedure. Ionic contrast (MD-76,

20 Mallinckrodt Medical Inc) was used throughout the experiment. Images were captured at a frame speed of 30 frames/sec, and stored digitally. A segment of contrast-filled guiding catheter was included in every frame, for the purpose of calibration. Calibration was performed using the known diameter of the contrast-filled guiding catheter as the reference segment, to avoid

25 error due to magnification. Coronary artery diameter measurements were made using a validated computerized edge-detection system (8). The midpoint of the injured segment was used for calculation of coronary artery diameter. For each analysis, coronary artery diameter measurements were performed in 3 consecutive end-diastolic frames, and the results averaged.

Measurements were performed by an independent observer blinded to the treatment group of the vessels.

Immunohistochemistry

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The animals were euthanized at 4 weeks. Under general anesthesia as described above, exsanguination was performed with replacement by 1 l of 0.9 % NaCl solution. The heart was perfusion-fixed in vivo with 2 l of 10 % buffered formalin at 200 mm Hg pressure. The heart was then removed, and
10 the coronary arteries were harvested immediately. From the injured segment (identified in relation to side branches), serial sections of 3-5 mm were made, and stored in 10 % buffered formalin solution. The sections were then treated with incremental concentrations of alcohol followed by treatment with xylene and paraffin. Slices of 6 μ m thickness were prepared, and stained with
15 Verhoeff's stain for assessment of tissue response to injury. For each injured segment, 2 slices demonstrating maximal neointima response were selected for immunohistochemistry, and the results obtained from analysis of the cross sections were averaged. The % of reendothelialization and, the % of endothelial nitric oxide synthase (eNOS) expression were calculated as
20 follows: (the total length of the luminal surface staining positively / the perimeter of the lumen) x 100, respectively. Analysis was performed by an independent examiner with no knowledge of the treatment groups to which the sections belonged. For lectin immunohistochemistry, the 6 μ m slices were first treated with hydrogen peroxide and methanol to block endogenous
25 peroxide, incubated with the *Dolichos biflorus* agglutinin (Sigma Chemical Co.) followed by treatment with 3,3'-diaminobenzidine (Vector Laboratories) and, subsequently counter-stained with hematoxylin. For immunohistochemistry of eNOS expression, after blocking of endogenous peroxide and non-specific antibodies, the slices were treated serially with the primary
30 mouse anti-eNOS antibody (Bio/Can Scientific), the secondary goat anti-

mouse antibody (Vector Laboratories), incubated with avidin-biotin (Vector Laboratories), treated with 3,3'-diaminobenzidine (Vector Laboratories) and finally counter-stained with hematoxylin. For both immunohistochemical examinations, normal porcine carotid artery slices were used as positive
5 controls; whereas slices obtained from the injured coronary arteries and stained only with hematoxylin were used as negative controls.

Statistical analysis

10 Values are expressed as mean \pm SD. Comparison of basal coronary artery diameter among the 3 groups was made using the one-way analysis of variance test. Comparisons between basal coronary artery diameter and coronary artery diameter following infusion of vasoactive agents were made with two-tailed Student's t-tests. The Kruskal-Wallis test was used for
15 comparison of lectin and eNOS expression among the 3 treatment groups. Linear relationships between lectin expression and response to Ach, and between eNOS expression and response to Ach were analyzed with Pearson correlation coefficients. Values were considered to be statistically significant if $p < 0.05$.

20

Results

There were no significant differences in basal coronary artery diameter (2.53 ± 0.6 mm for 17-beta estradiol, 2.79 ± 0.35 mm for PTCA only, and
25 2.77 ± 0.44 mm for vehicle groups respectively, $p < 0.4$) among the 3 treatment groups. The extent of morphologic tissue injury (9) among the groups was similar. No changes in heart rate, ECG, or blood pressure were noted during the local delivery or during intracoronary infusion of vasoactive agents.

30

Response of PTCA only group to Ach

Compared to the basal coronary artery diameter, there were no significant changes in coronary artery diameter following intracoronary infusion of 10^{-7} M and 10^{-6} M concentrations of Ach (Table). At a concentration of 10^{-4} M a significant vasoconstrictive response was noted ($p < 0.02$). A marked vasoconstrictive response was observed at a concentration of 10^{-4} M ($p < 0.0001$) (Figure 3). The vasoconstriction was completely reversed upon administration of the endothelium-independent vasodilator nitroglycerin. Coronary diameter increased from 1.8 ± 0.48 mm after 10^{-4} M Ach, to 2.5 ± 0.28 mm following nitroglycerin ($p < 0.01$; $p = 0.2$ for post-nitroglycerin vs basal diameter).

Response of vehicle treatment group to Ach

Compared to the basal coronary artery diameter, 10^{-7} M Ach did not change coronary artery diameter in the vehicle treatment group (Table 3). A trend towards significant vasoconstriction was noted with 10^{-6} M Ach ($p = 0.06$). Significant vasoconstriction was produced by 10^{-5} M ($p < 0.02$), and at 10^{-4} M ($p < 0.001$) Ach infusion respectively (Figure 3). Nitroglycerin completely reversed the vasoconstriction, returning the arteries to their basal diameter (from 1.89 ± 0.51 mm after 10^{-4} M Ach, to 2.69 ± 0.52 mm following nitroglycerin [$p < 0.004$; $p = 0.7$ for post-nitroglycerin vs basal diameter]).

Response of 17-beta estradiol treated group to Ach

In the vessels treated with local delivery of 17-beta estradiol no significant vasoconstrictive response to Ach occurred at any concentration used (Table) (Figure 3). A mild and statistically nonsignificant increase in coronary artery diameter was observed following administration of nitroglycerin: from $2.28 \pm$

0.61 mm after 10^{-4} M Ach to 2.61 ± 0.48 mm after nitroglycerin ($p = 0.4$; $p = 0.8$ for post-nitroglycerin vs basal diameter).

Immunohistochemistry

5

Immunohistochemical analyses were performed 4 weeks after PTCA on all 9 animals. Three arterial segments were lost/damaged during harvesting of the samples (2 of PTCA only group, and 1 of vehicle group). Significant differences were seen among the 3 treatment groups in the extent of reendothelialization, as assessed by immunohistochemical analysis with the lectin *Dolichos biflorus* agglutinin (Figure 4). Reendothelialization was noted to a greater extent in vessels treated with local delivery of 17-beta estradiol compared to the other 2 groups (90.6 ± 5.5 % for 17-beta estradiol 71 ± 6.8 % for PTCA only, and 72.8 ± 4.9 % for vehicle, $p < 0.0005$). Endothelial nitric oxide synthase expression was also higher in vessels treated with 17-beta estradiol (35.6 ± 11.8 % for 17-beta estradiol 9.4 ± 3.9 % for PTCA only, and 9.2 ± 4.0 % for vehicle, $p < 0.0005$) (Figure 5). No significant differences in immunohistochemical analyses were observed between vessels treated with vehicle or PTCA only.

20

We proceeded further to analyze whether a linear relationship between reendothelialization and the response to Ach could be demonstrated. A significant inverse correlation was noted between reendothelialization as assessed by immunohistochemistry with the lectin *Dolichos biflorus* agglutinin and the response to Ach ($r = 0.48$, $p < 0.02$) (Figure 6). An even stronger inverse linear correlation was observed between eNOS expression and the response to Ach ($r = -0.58$, $p < 0.005$).

25

Conclusions

This study demonstrates for the first time that local delivery of 17-beta estradiol immediately following PTCA enhances subsequent
5 reendothelialization and endothelial function at the site of injury. Besides its critical role in the regulation of vascular tone, the normal endothelium functions as an effective barrier between blood elements and underlying vascular smooth muscle cells. Endothelium-derived nitric oxide (NO) is a potent vasodilator, inhibits monocyte adherence and platelet aggregation
10 and adhesion (10), vascular smooth muscle cell migration (11) and proliferation (12).

PTCA is associated with arterial injury and damage to the endothelium (3). Following arterial injury, varying rates of reendothelialization have been
15 reported. Reendothelialization rates of 81 % (13), and even lower rates of < 50 % (14) following arterial injury have been observed. In a study of specimens of restenotic lesions obtained by atherectomy in humans, no endothelial cells could be demonstrated (15). In the present study, local treatment with 17-beta estradiol was followed by nearly complete
20 reendothelialization (90.6 ± 5.5 %), which was significantly greater than that observed in the groups not treated with 17-beta estradiol. Estrogen receptors have been identified in human coronary artery and umbilical vein endothelial cells (16), and when bound to estrogen are capable of regulating protein synthesis by altering transcription rates (17). In cell culture assay of human
25 umbilical vein endothelial cells, treatment with 17-beta estradiol markedly increased both cell migration and proliferation (18). Therapy with subcutaneously implanted 17-beta estradiol pellets significantly enhanced reendothelialization following arterial injury (6). The capacity of 17-beta estradiol to increase vascular endothelial growth factor synthesis (19) and
30 the effort of 17-beta estradiol on basic fibroblast growth factor may be

responsible for the enhanced reendothelialization. Vascular endothelial growth factor treatment is known to promote reendothelialization in vivo (20). In human umbilical vein and coronary artery endothelial cell culture experiments, treatment with 17-beta estradiol enhanced the release and phosphorylation of basic fibroblast growth factor (21,22). It has been shown that administration of basic fibroblast growth factor in vivo stimulates reendothelialization following arterial injury in rats (23). Another mechanism by which 17-beta estradiol could possibly influence extent of reendothelialization is by inhibition of apoptosis of injured endothelial cells: a 50 % decrease in apoptosis was seen with 17-beta estradiol treatment of human umbilical vein endothelial cells exposed to tumor necrosis factor- α (24). It is noteworthy that increased expression of tumor necrosis factors is known to occur following balloon injury (25)-

Impaired endothelial function, as in atherosclerosis (26) or following experimental inhibition of NO (27), has been associated with a paradoxical constrictive response to Ach. This paradoxical response to Ach could be modified by treatment with estrogen. In humans, 17-beta estradiol administered intravenously (28) or by continuous intracoronary infusion (29), attenuated the vasoconstrictive response to Ach and also inhibited the Ach-induced increase in coronary resistance and decrease in coronary blood flow. The regulatory effect of 17-beta estradiol on eNOS that we observed may be responsible for the beneficial effects on endothelial function, as vascular response to Ach is closely related to eNOS expression (30,31). In support of this notion, a strong inverse linear relationship was seen between the vascular response to Ach and eNOS expression (Figure 4). The ability of estrogen to induce nitric oxide synthase was first identified during gestation in guinea pigs (32). Induction of eNOS function by 17-beta estradiol has been subsequently demonstrated to be accompanied by increased eNOS protein and mRNA expression (33,34). Increased circulating NO levels

Gender differences in the endothelium-dependent vasodilation by 17-beta
30 estradiol have been noted (39). In our study, a majority of animals were

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30 estradiol have been noted (39). In our study, a majority of animals were

mates and a significant beneficial effect of 17-beta estradiol was noted in all the animals studied, irrespective of sex. Thus, local delivery of 17-beta estradiol appears to be effective in males as well as females. There is evidence to suggest that the simultaneous administration of progesterone
5 reduces NO levels induced by 17-beta estradiol (35), this issue was, however, beyond the scope of the present study.

We conclude that a single dose of 17-beta estradiol delivered locally following balloon injury can significantly improve reendothelialization and
10 enhance endothelial function at the injured site as late as 1 month following injury. Besides the beneficial vascular effects of improved endothelial function, this observation may be of particular importance following balloon angioplasty as improved endothelial function is known to be associated with decreased neointima formation in the injured area (20,40). This approach
15 merits further study, with a view to potential clinical value in the prevention of vascular dysfunction and restenosis following PTCA.

Formulations

20 The formulations may include estradiol or a derivative thereof and any pharmaceutically acceptable vehicle. Since estradiol is a lipophilic molecule, such vehicle would ideally include a solvent component. Such a solvent component includes molecules such as propylene glycol, ethanol, and detergents, for example PluronicTM. The formulations may take the form of
25 a liquid, a suspension, a semi-solid or a thermoreversible composition which may form a layer over the endothelium. The formulations may further be included in or used as a coating for a device such as a stent, or be part of any similar device that can be left *in-situ* upon angioplasty or vascular surgery.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, these embodiments can be modified at will, without departing from the spirit and nature of the subject invention. Such modifications are within the scope of the present invention as defined in the

5 appended claims.

Characteristics	Male	Female	p value
Restenotic index	1.2 ± 0.59	1.37 ± 0.45	> 0.1
Neointima area (mm ²)	0.51 ± 0.34	0.25 ± 0.15	> 0.1
Neointima/Media area	0.78 ± 0.55	0.32 ± 0.16	> 0.1
% neointima	14.93 ± 10.68	8.29 ± 3.72	> 0.1
% stenosis	18.93 ± 13.39	11.09 ± 5.16	> 0.1

Table 3: Response to Intracoronary Acetylcholine

Ach*	Diameter-basal (mm)	Diameter-post Ach (mm)	p value
<i>PTCA group</i>			
10 ⁻⁷ M	2.79 ± 0.35	2.65 ± 0.35	0.4
10 ⁻⁶ M	2.79 ± 0.35	2.54 ± 0.32	0.1
10 ⁻⁵ M	2.79 ± 0.35	2.3 ± 0.35	0.02
10 ⁻⁴ M	2.79 ± 0.35	1.8 ± 0.48	0.0001
<i>Vehicle group</i>			
10 ⁻⁷ M	2.77 ± 0.44	2.6 ± 0.41	0.4
10 ⁻⁶ M	2.77 ± 0.44	2.33 ± 0.5	0.06
10 ⁻⁵ M	2.77 ± 0.44	2.24 ± 0.47	0.02
10 ⁻⁴ M	2.77 ± 0.44	1.89 ± 0.51	0.001
<i>17-beta estradiol group</i>			
10 ⁻⁷ M	2.53 ± 0.6	2.46 ± 0.58	0.8
10 ⁻⁶ M	2.53 ± 0.6	2.38 ± 0.58	0.6
10 ⁻⁵ M	2.53 ± 0.6	2.36 ± 0.59	0.6
10 ⁻⁴ M	2.53 ± 0.6	2.28 ± 0.61	0.4

* acetylcholine

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WHAT IS CLAIMED IS:

1. The use of 17- β estradiol or a derivative thereof in the making of a medication or a device for in-situ administration in the lumen of a blood vessel having suffered vascular injury, at the injured site, for improving reendothelization and vascular endothelial function in a patient.
2. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 $\mu\text{g/Kg}$ of patient's body weight.
3. The use, as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 50 $\mu\text{g/Kg}$ of patient's body weight.
4. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 30 $\mu\text{g/Kg}$ of patient's body weight.
5. The use as defined in any one of claims 1 to ~~5~~⁴, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).
6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.
7. The use as defined in Claim 4, where 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 March 2001 (29.03.2001)

PCT

(10) International Publication Number
WO 01/21157 A2

(51) International Patent Classification⁷: **A61K 31/00**

TANGUAY, Jean-François [CA/CA]; C.P. 323, succ. Mont-Royal, Monreal, Quebec H3P 3C5 (CA).

(21) International Application Number: PCT/CA00/01132

(22) International Filing Date:
21 September 2000 (21.09.2000)

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(25) Filing Language: English

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(26) Publication Language: English

(30) Priority Data:
2,282,982 21 September 1999 (21.09.1999) CA
2,300,246 9 March 2000 (09.03.2000) CA

(71) Applicant (*for all designated States except US*): INSTITUT DE CARDIOLOGIE DE MONTREAL [CA/CA]; 5000 Bélanger Street East, Montreal, Quebec H1T 1C8 (CA).

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

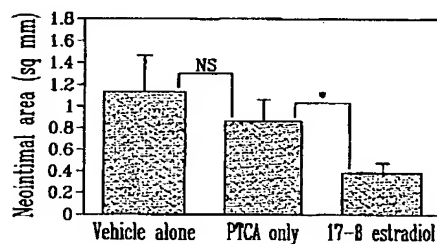
(75) Inventors/Applicants (*for US only*): CHANDRASEKAR, Baskaran [IN/IN]; 2, Justice Ramanujam Road, Dr. Radhakrishnan Nagar, Chennai 600 041 (IN).

Published:

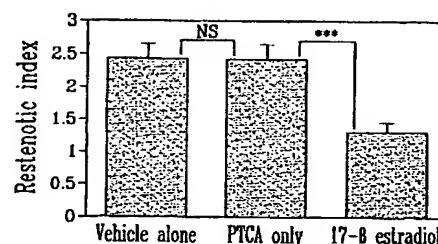
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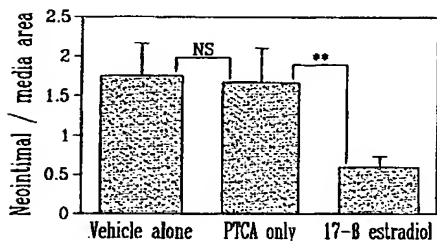
(54) Title: LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY



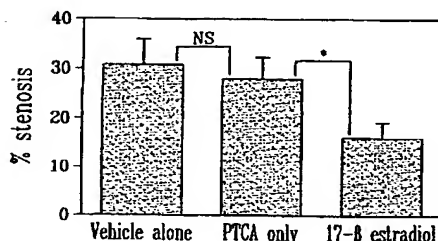
A



C



B



D

(57) Abstract: The cardioprotective effects of estrogen are well recognized. In *in vitro* experiments, and upon systemic administration, 17-beta estradiol has shown to inhibit vascular smooth muscle cell proliferation and intima hyperplasia and to improve vascular endothelium function, after vascular injury. We hypothesized that locally delivered 17-beta estradiol could prevent restenosis. Compositions are use of 17-beta estradiol for *in-situ* administration at a vascular injured site are objects of the present invention.

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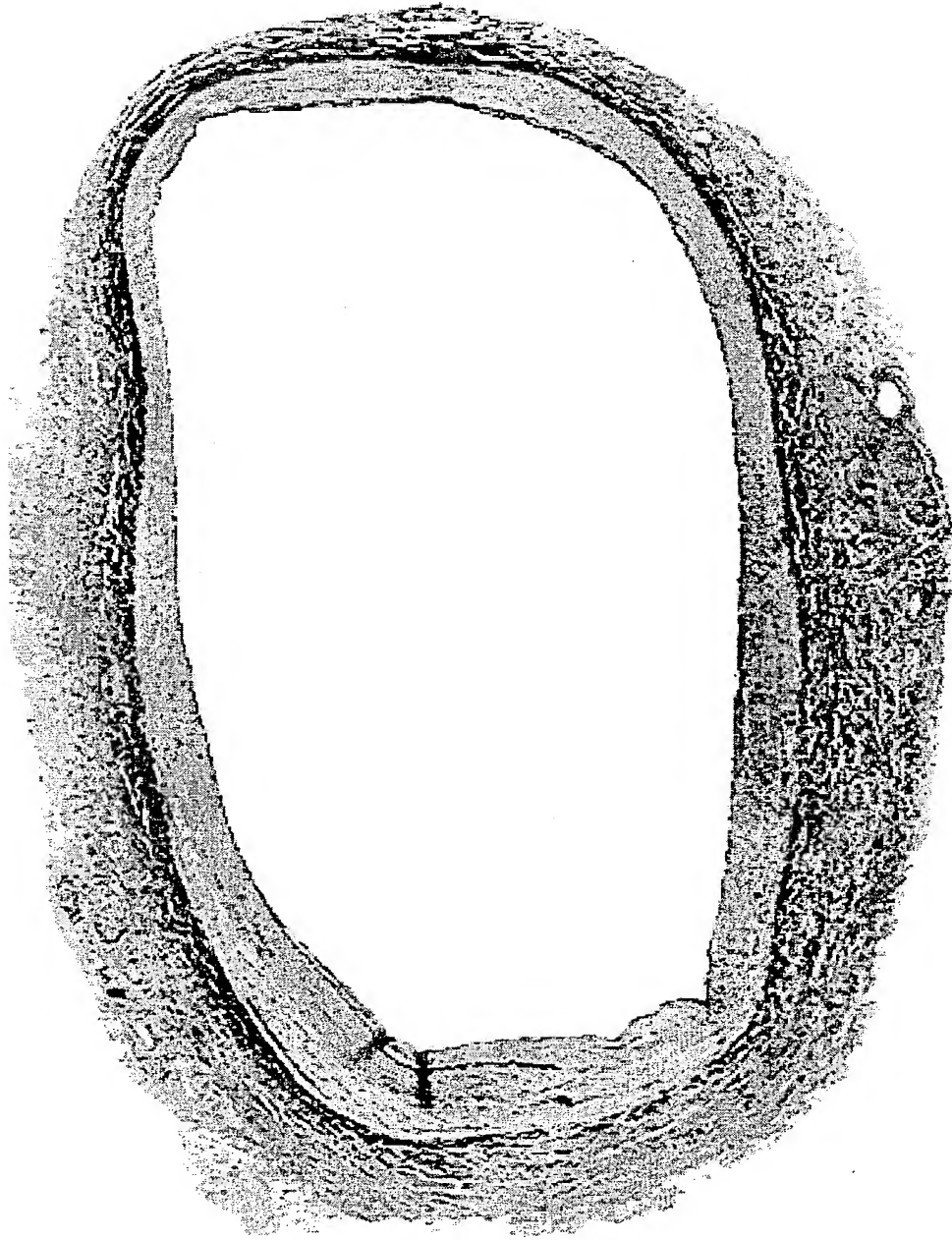


FIG. 1A

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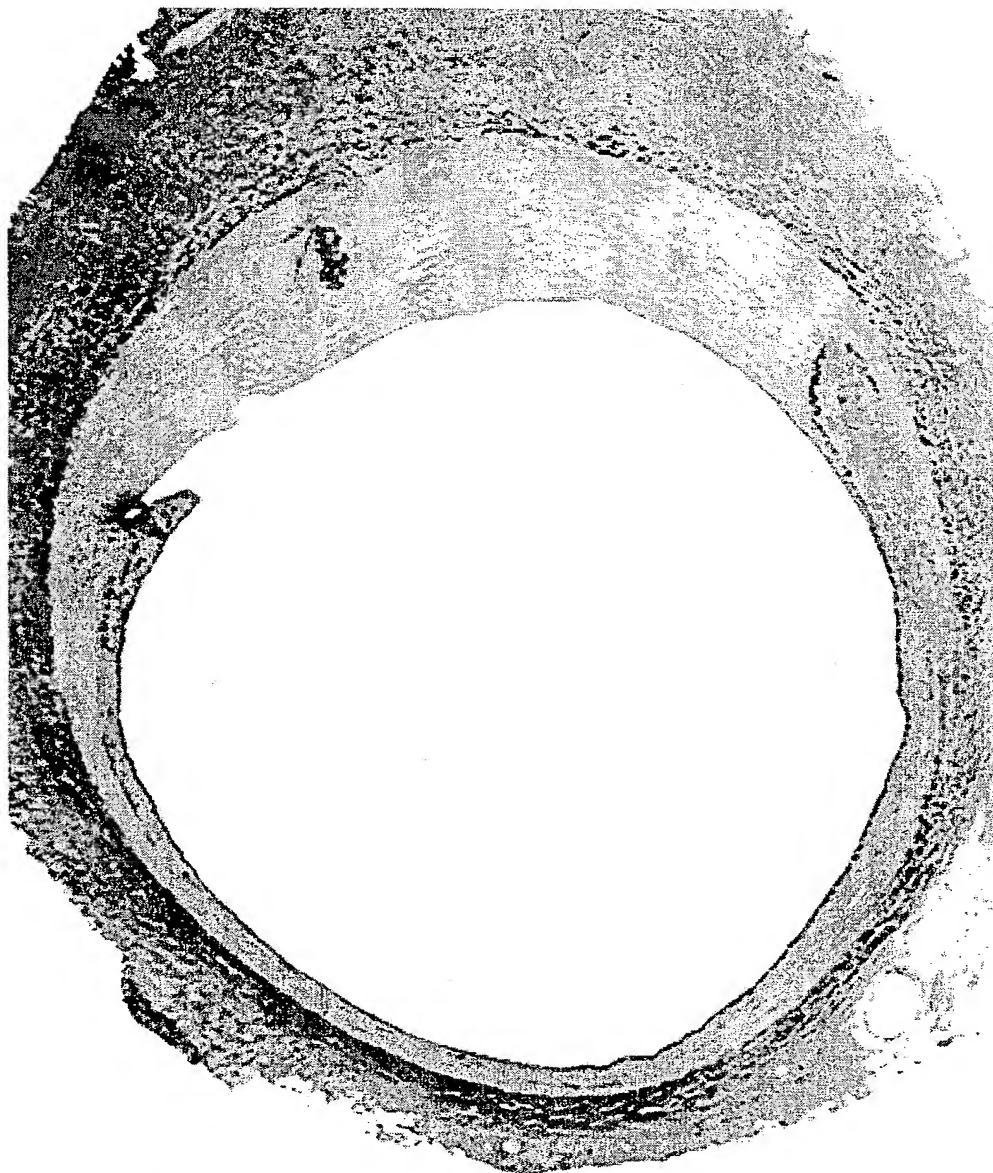
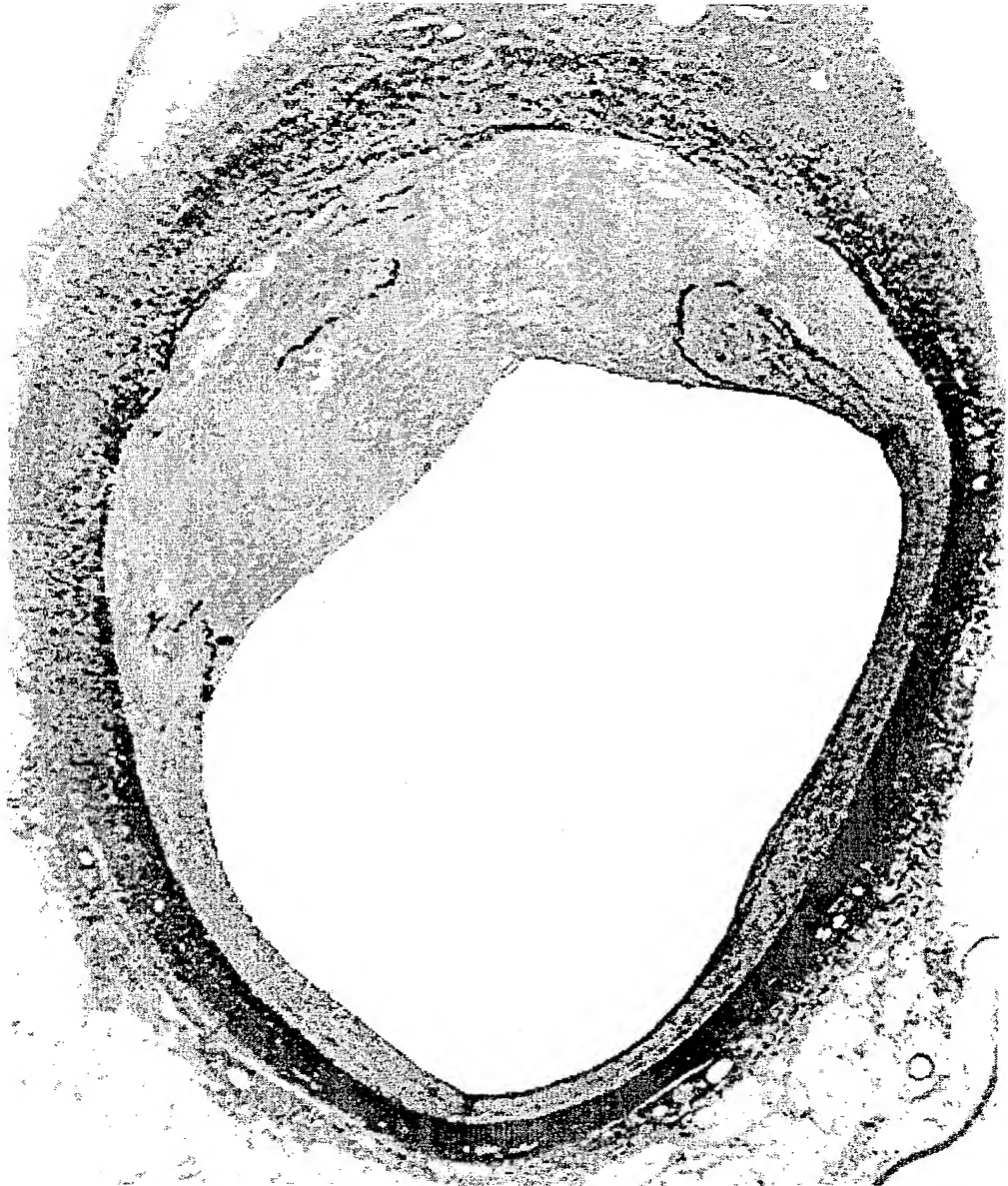


FIG. 1B

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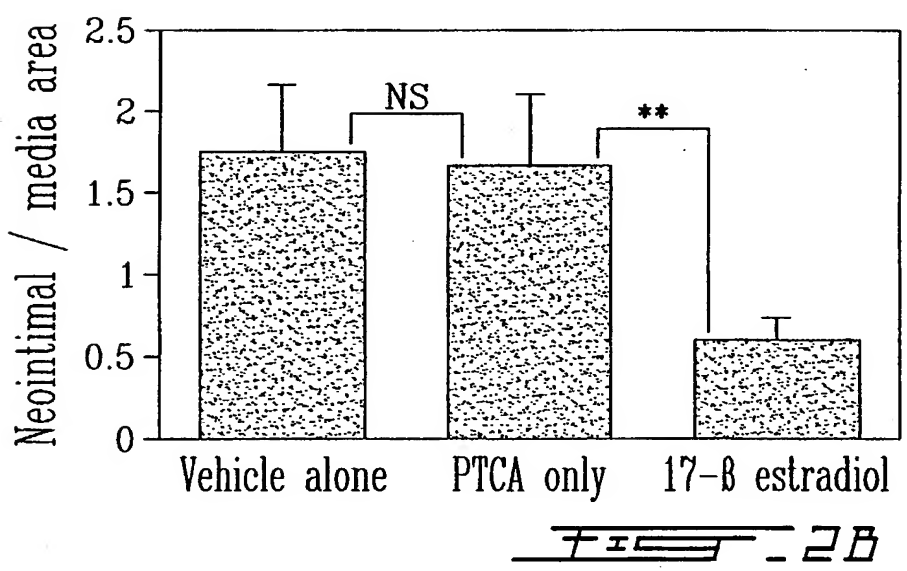
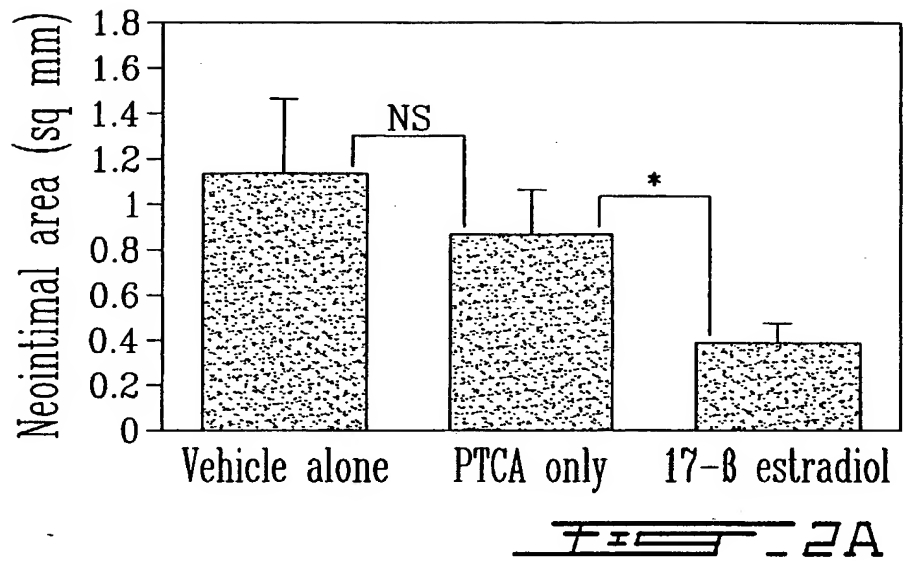
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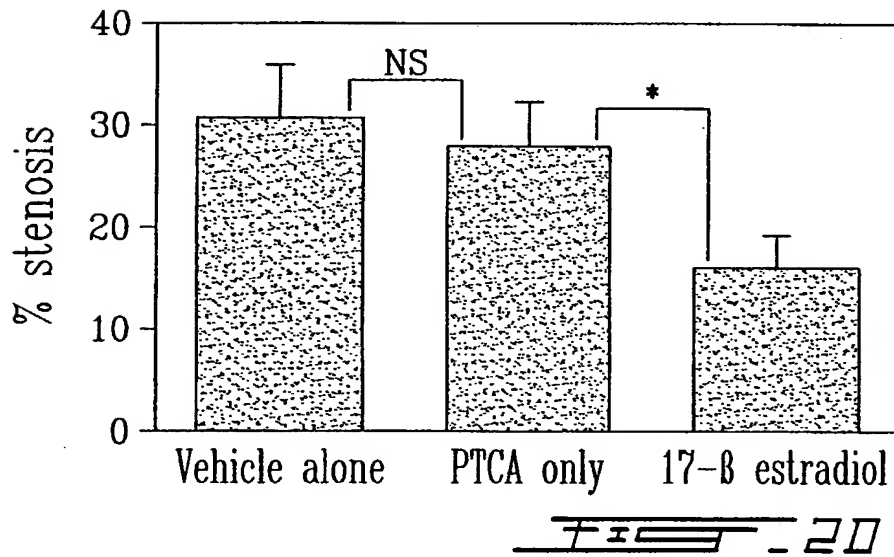
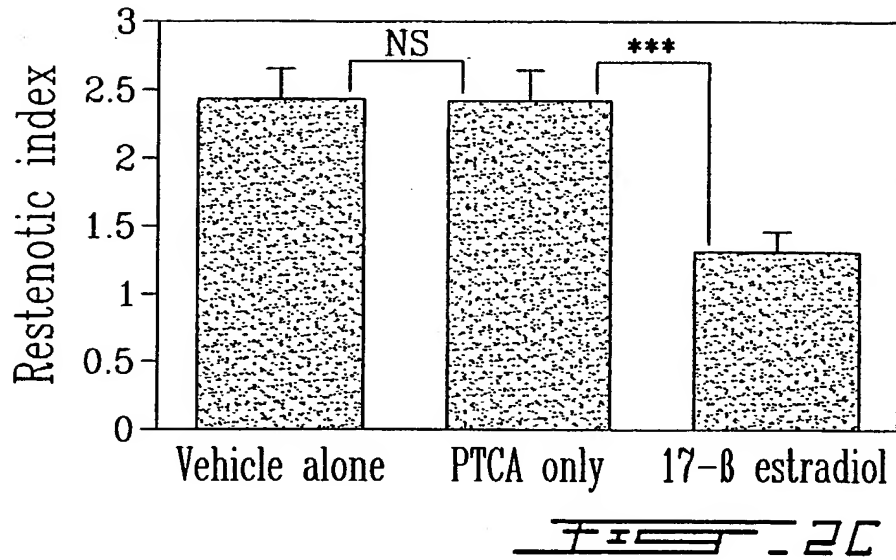
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FIG. 3A

FIG. 3B

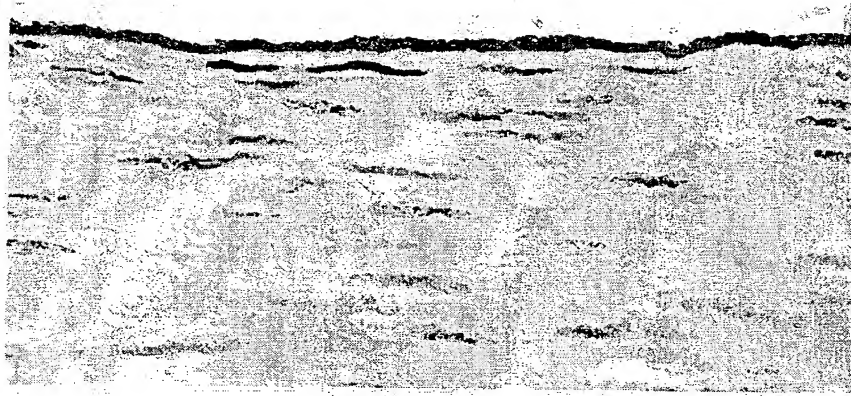
FIG. 3C


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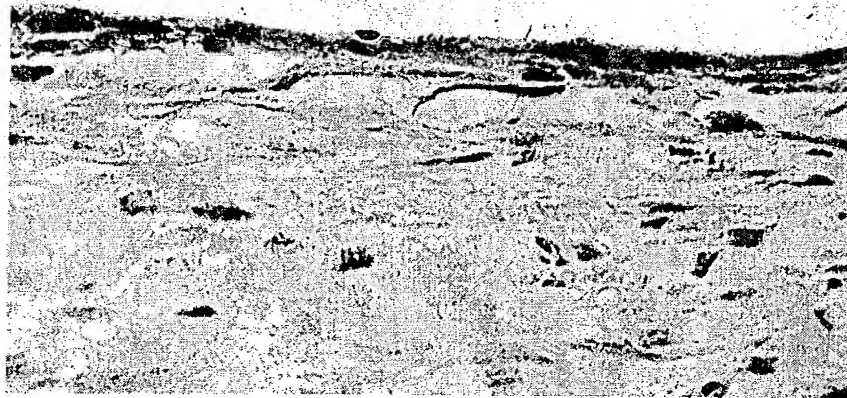
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 4A



 4B



 4C

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FIG. 5A

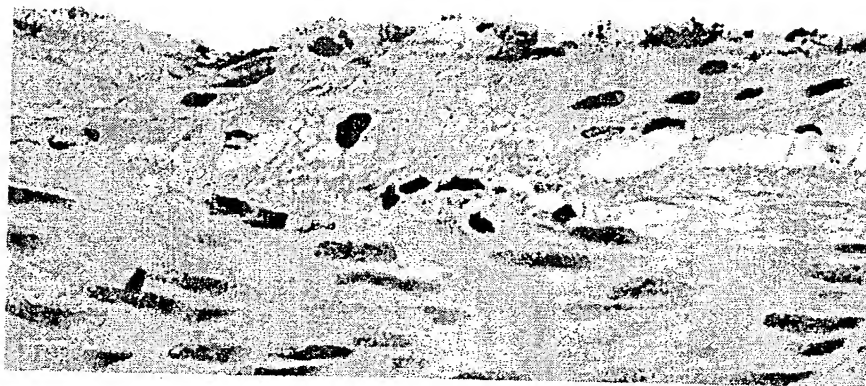


FIG. 5B

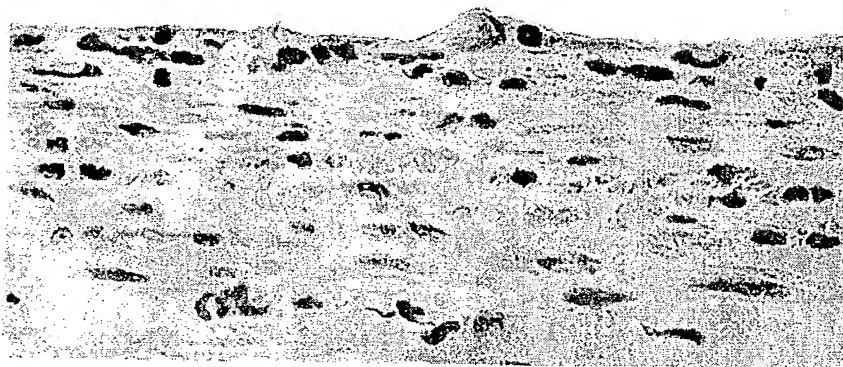
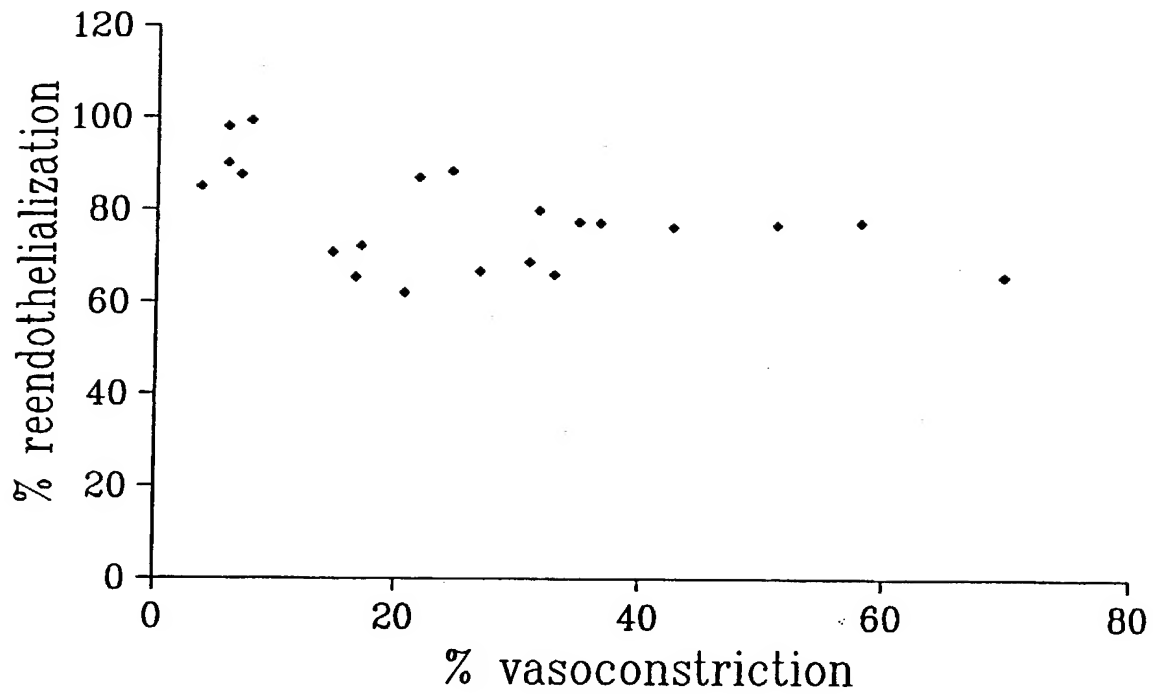
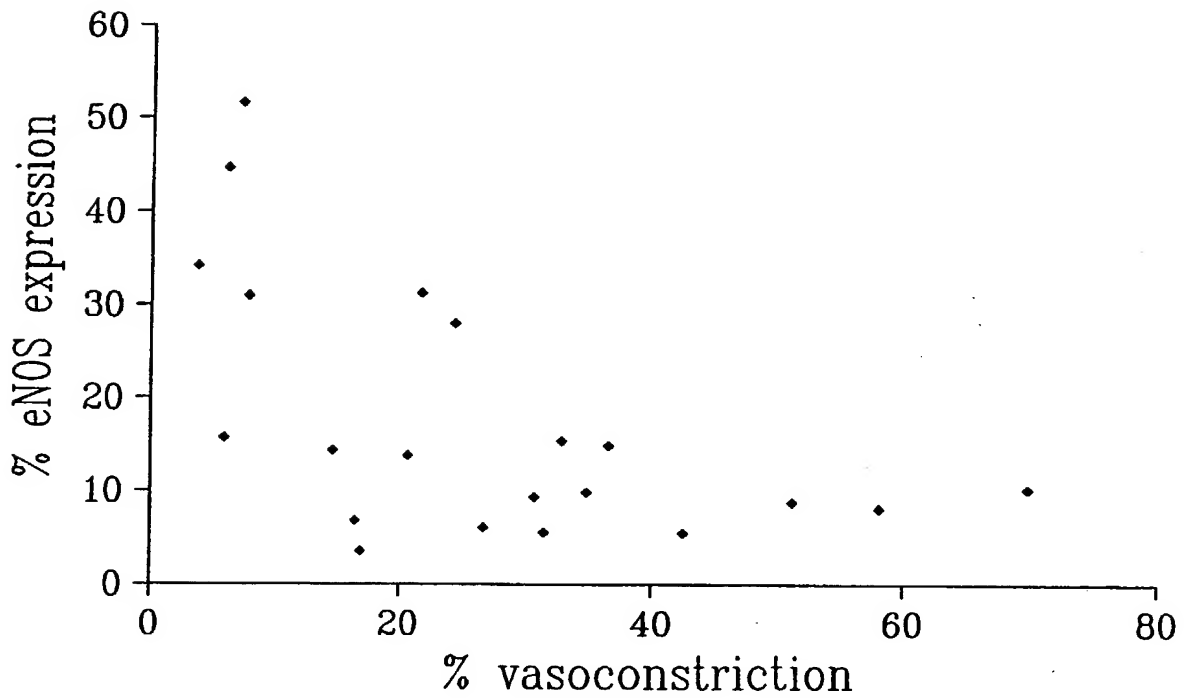


FIG. 5C

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BA



BB

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET
NUMBER
410718.90395

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA
HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER
VASCULAR INJURY

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as U.S. Patent Application Serial Number ____
on ____,
as amended on ____ (if applicable).

☒ was filed as a PCT international application number PCT/CA00/01132 on 21 Sept 2000
as amended under PCT Article 19 on 11/19/01 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

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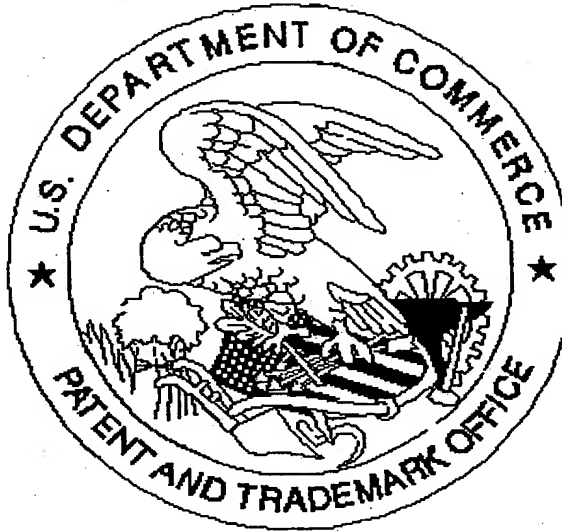
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COUNTRY (If PCT Indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/CA00/01132	21 Sept 2000	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
CA	2,282,982	21 Sept 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
CA	2,300,246	09 March 2000	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)				ATTORNEY DOCKET NUMBER 410178.90395	
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201	FULL NAME OF INVENTOR	FAMILY NAME CHANDRASEKAR	FIRST GIVEN NAME Baskaran	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY Dr. Radhakrishnan Nagar	STATE OR COUNTRY Chennai <i>INX</i>	COUNTRY OF CITIZENSHIP India	
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	RESIDENCE & CITIZENSHIP	CITY Montreal	STATE OR COUNTRY Quebec <i>CAX</i>	COUNTRY OF CITIZENSHIP Canada	
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203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY	
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SIGNATURE OF INVENTOR 201		SIGNATURE OF INVENTOR 202		SIGNATURE OF INVENTOR 203	
DATE <i>[Signature]</i> 29/5/2002		DATE <i>[Signature]</i> 29/5/2002		DATE	

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